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14. ABSTRACT

The purpose of our study is to understand the role of novel polarity regulators mammary gland development and their ability to cooperate with oncogenes in tumorogenesis within this gland. We are using mouse systems as well as an analysis of cell lines to understand the role of a particular gene, Scribble, in this process.
So far, I have been able to identify one human breast cancer cell line with little scribble expression. In a normal mouse cell line, comma-1D, we are doing further analysis as to the effects of scribble loss using RNAi technology. We have observed a morphological change, with a loss of e-cadherin, as well as a mild proliferative change in scribble knockdown cells. We have also observed a change in lineage specific cytokeratins in these cells.
This data is significant as it possibly demonstrates the role of a polarity gene the differentiation of the breast as well as in tumors with concomitant growth changes to the affected tissue.

15. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
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Table of Contents

Introduction

We are interested in understanding the role of polarity genes in the development of the mammary gland as well as in breast cancers. This information is of importance as one of the hallmarks of the breast, as well as all epithelial tissues is the arrangement of polarized secretory cells around a hollow lumen. This polarized organization is lost early in the development of epithelial hyperplasias. To further provide evidence for the importance of polarity genes, genetic screens were done in drosophila that indicate that a loss of some polarity genes (Scribble, lgl, dlg) cooperate with some oncogenes (Rasv12, Cyclin E) (Pagliarini RA et al., Brumby AM et al.) to promote overgrowth of a tissue as well as invasion. We have selected some of these genes as candidates in breast cancer and are studying them both alone and in combination with known oncogenes to determine their role in mammary epithelial cells in cell culture as well as in the mouse mammary gland. In this summary I will focus on the work done with scribble.

Body

Using the mouse mammary epithelial cell line, Comma-1D (Danielson KG et al.), a relatively normal cell line derived from the breast of a midpregnant balb/c female, the knockdown of scribble was achieved (Figure 1a) using an MSCV retroviral shRNA embedded in a mir30 context (Silva JM, et al.). We have two shRNAs that are working in these cells, one in the 3'UTR and the other targeting the final LRR domain. Using the 3'UTR shRNA, the cells' morphology (figure 1b) is altered with the cells becoming flatter and not packing as tightly as the control cells.

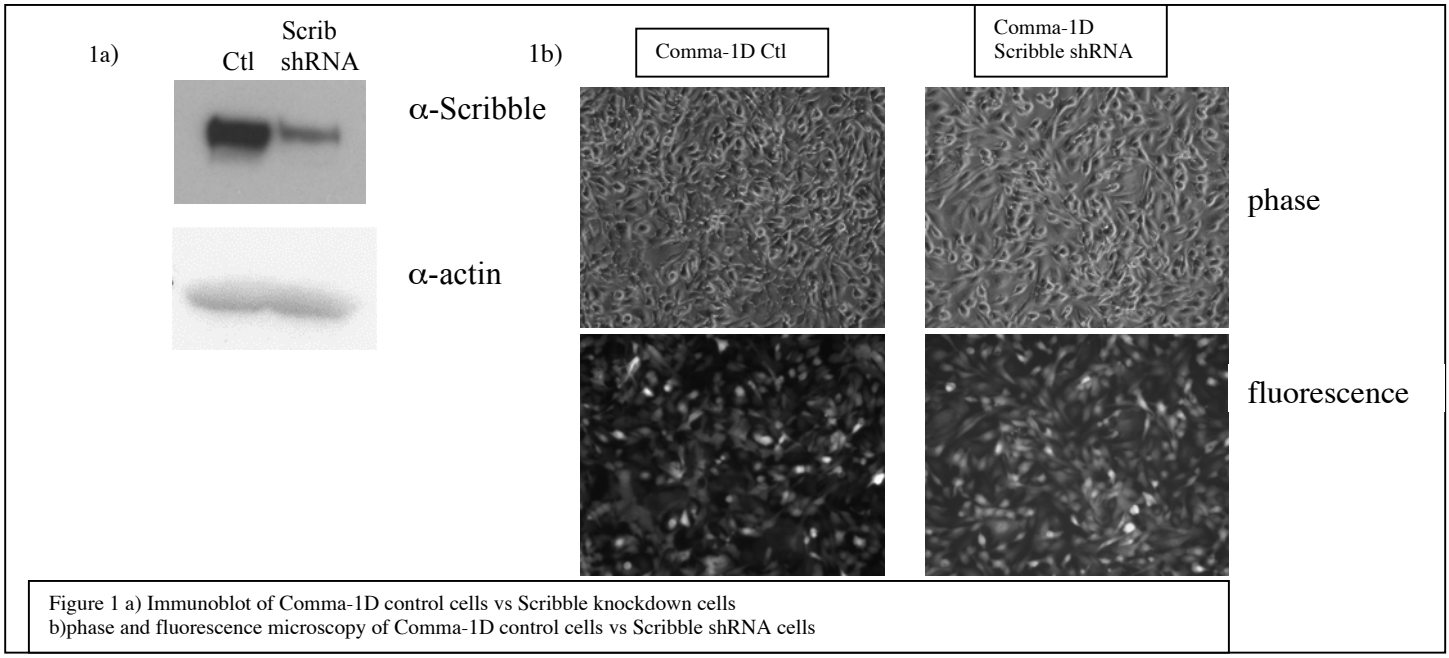
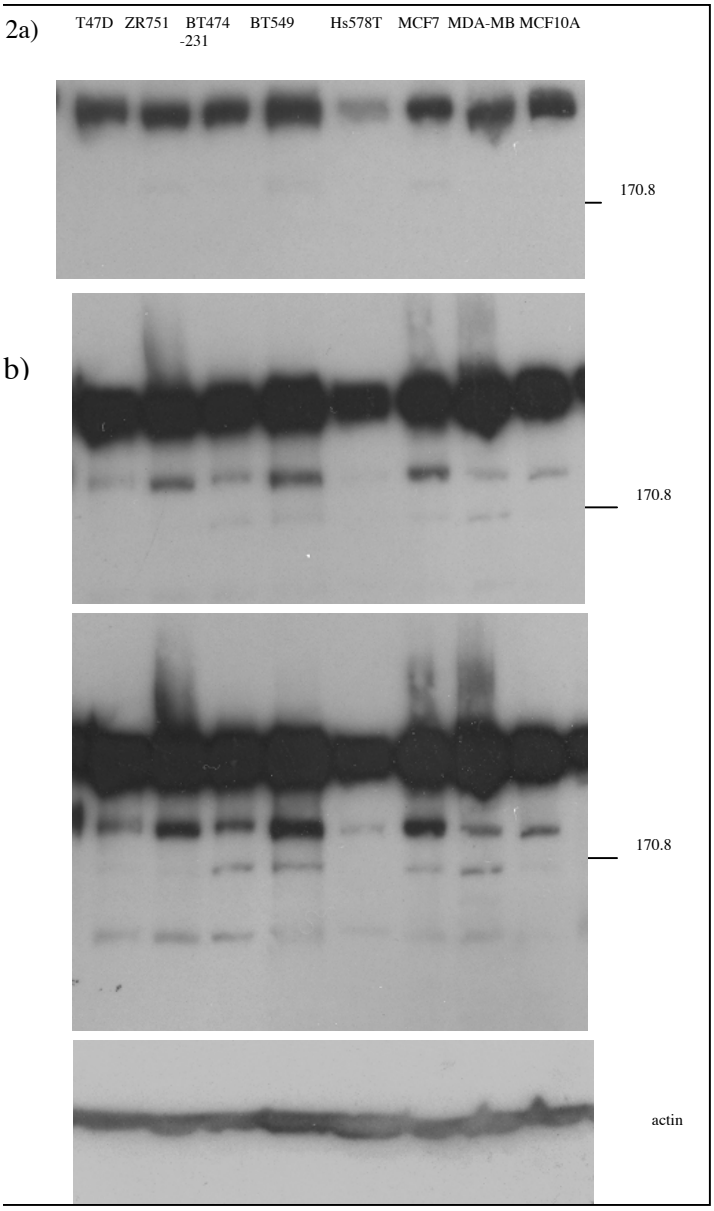


Figure 1 a) Immunoblot of Comma-1D control cells vs Scribble knockdown cells
b)phase and fluorescence microscopy of Comma-1D control cells vs Scribble shRNA cells

The comma-1D cells were used in order to have a continuous source of cells for future transplantation into mice as well as for in-vitro analysis. This is their benefit over primary cells, which cannot be maintained over multiple passages. Additionally, these cells upon transplantation give rise to relatively normal, though mildly hyperplastic glands.

We did a comparison of 8 human mammary and breast cancer cell lines. There is apparently a range of expression levels amongst these cells with HS578T cells having the lowest expression (Figure 2a). There are also changes in the lower bands on the immunoblot that may correlate with other isoforms of scribble. The relative amounts of these bands are different amongst the different cells lines. (Figure 2b higher exposure)



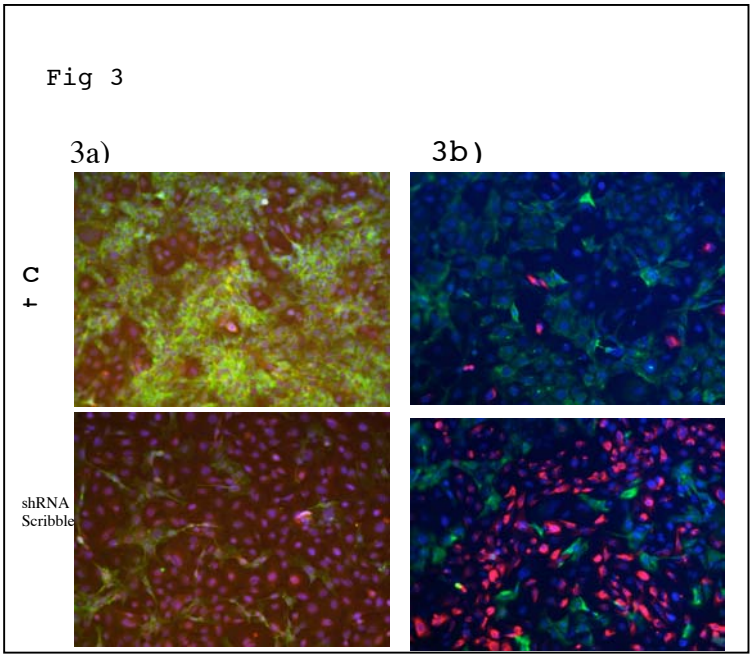


Fig 2: Immunoblot of scribble in breast cancer cell lines
2a) Breast cancer cell lines probed anti scribble antibody, Low exposure
2b)higher exposure of 2a.

Fig 3:IF of cytokeratin 6 and 18, E-cadherin in Comma-1D control vs Scribble shRNA
a)E-cadherin (green)
b)cytokeratin 6 (red) and cytokeratin 18 (green)

In order to evaluate the morphological differences between the cells we looked at E-cadherin. We observed a decrease in E-cadherin (Figure 3a) Similar results have been obtained recently in two studies. In one (Navarro C. et al.) an analysis of clinical samples was shown to correlate the loss of scribble with an e-cadherin loss in lobular breast carcinomas. In the other study of MDCK cells showing that Scribble knockdown was associated with a loss of e-cadherin (Qin Y et al.).

In order to evaluate the differentiation status of the different populations we looked at Cytokeratin 6 and 18 (Fig 3b) as a marker of hyperproliferation with a natural or pathological turnover as well as in basal cells. Cytokeratin 18 is a marker of the epithelial cells. It is apparent that there is an increase in the number of CK6 positive cells with a concomitant decrease in CK18. These results will lead us to follow up the differentiation status of these cells. Are they more basal? Less epithelial? This analysis is being carried out in these cells by looking at other markers of basal cells, i.e., SMA. Additionally, studies of primary mammary epithelial cells with a knockdown of scribble are being to looking for similar effects in primary cells.

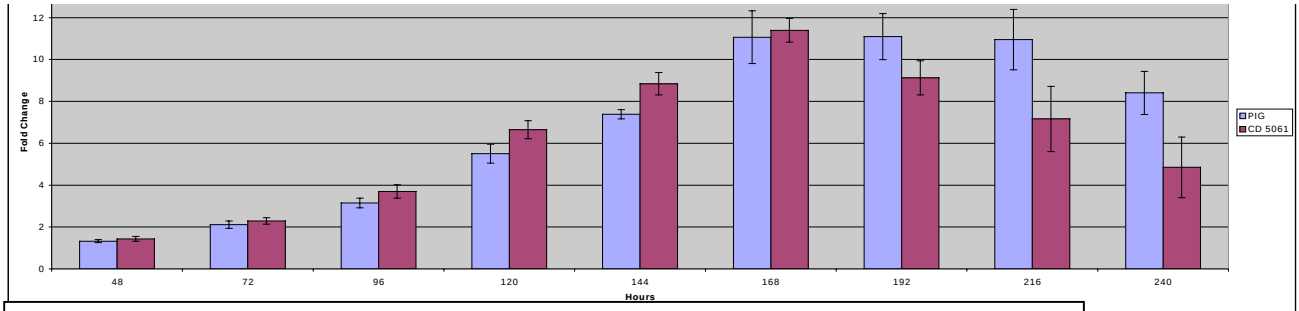


Fig 4: Proliferation assay. Comma-1D ctl (PIG) vs Scrib shRNA (CD 5061)

Considering that cytokeratine 6 is a marker of hyperproliferation (Smith GH, et al.) and that scribble loss in some tissues resulted in overgrowth of that tissue, we started to analyze the proliferation status of the knockdown cells. It appears that Scribble knockdown cells have a slight proliferative increase over control cells. This was the case in wing discs, (Zeitler J et al.) in which they saw an overproliferation in the wing disc as well as a loss of polarization. However, after 7 days in culture the cells either die off or lose their adhesiveness and there is an, as of yet, unexplained decrease in the number of cells using a proliferation assay. (Figure 4) With RasV12 overexpression in Scribble knockdown cells there is a cooperation compared to RasV12 alone. However, this data is not complete.

(Note: the data represented in Fig 4 is representative of only one experiment that contained 6 replicates. The assay was done as follows: 1000 cells/well were plated for each genotype in a 96 well plate. 1 plate was fixed at 24 hr intervals thereafter in gluteraldehyde. The cells were then stained with methylene blue and extracted with 0.1 N HCl. They were then read on a plate reader at 650 nM. Each value was corrected to day 1 and fold change over 24 hrs was plotted.)

These comma-1D cells were then used to transplant to the cleared fat pad of 3 week old mice. When comparing the Ras alone to Ras Scribble KD, tumors formed in both groups with a very short latency 12-15 days with some segregation between the two groups. It is possible that the difference may be in metastases from the primary tumor and we are following this up with histology of the lungs looking for metastasis. Overt, gross metastasis were visible in both groups but their onset hasn't been well characterized. However, this difference was very mild and repeats are in progress with a second batch of cells.

Key Research & Training Accomplishments

- Knockdown in normal mouse and human cell lines of scribble with two different stable retroviral shRNAs
- Morphological changes in Commad-1D cells with scribble knockdown
- Cytokeratin 6 increases, E-Cadherin decreases in the scribble knockdown cells
- Mild Proliferation increase in scribble knockdown cells
- Cooperative proliferation increase in Ras Scribble knockdown cells (repeats in progress)
- All the tools necessary to achieve the Tasks presented in Statement of work are in place to begin addressing them with the promise of interesting results

Conclusions

The loss of scribble in a heterogenous population of mouse mammary epithelial cells apparently leads to an increase in a basal, hyperproliferation marker, cytokeratin 6. Basal carcinomas are an aggressive invasive form of invasive ductal carcinoma. Loss of scribble may allow for an expansion of the basal cell population that with a cooperative oncogene will cause an invasive ductal carcinoma of the basal form. This is an important question as little is understood of this disease. Additionally it was recently shown that scribble is lost in a large portion of lobular carcinomas of the breast. A hallmark of these tumors is their lack of E-cadherin. Hence, the model we are using may be useful in understanding this distinction between the different forms of carcinomas as our preliminary data gives a hint that scribble may act as a control over the differentiation status of mammary epithelial cells.

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